Exhibit D

NOVEL SYNTHETIC ANALOGUES OF IL-10 REGULATE THE BINDING OF NF-kappaB COMPLEXES TO p53 and IL-8 kappaB MOTIFS

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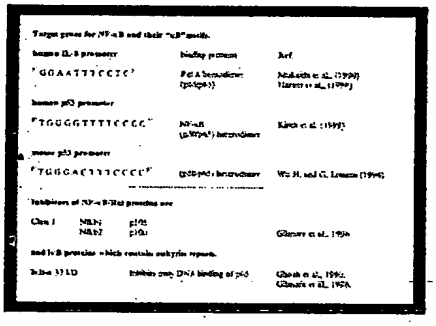
INTRODUCTION

NF-κB is a transcription factor involved in the regulation of inflammation and growth in both benign and malignant cells (Gilmore et al., 1996). We have recently shown that in hepato-carcinoma cells both IL-10 and IT9302 (-Ala-Tyr-Met-Thr-Met-Lys-Ile-Arg-Asn-), a nona-peptide homologous to IL-10's COOH terminal domain, is regulating NF-κB complexes by inducing NF-κB 50 and IκB-α and thereby inducing apoptosis (Gesser et al., 2002). In this study we tested whether IL-10, IT9302 and two novel IL-10 analogues are able to regulate NF-κB binding to specific "κB motifs" in monocytes as well as in a monocytic cell line U937. We constructed novel IL-10 analogues by modifying the amino-acid composition of IT9302, substituting single amino acids with natural or non-natural amino-acids.
IL-10 is known for inhibiting Nuclear Factor κB activation (Wang et al., 1995) and for stabilizing Inhibitory κB-α in monocytes (Shames et al., (1998). IL-10 is also blocking κB kinase activation and induced NF-κB p50/p50 in the monocytic cell line U937 (Schottelius et al., 1999).
Specific "κB motifs" are identified for IL-8: Mukaida et al., (1990), Harant et al., (1998) as well as for p53, Kirch et al., (1999).
We find that both IL-10 and IT9302 (in equimolar concentrations) suppress NF-κB binding to DNA, around 10 to 30 minutes after stimulation (Fig. 1).
We further find that IL-10, IT9302 and novel analogues also modify the binding of NF-κB to the IL-8 "κB motif" as well as to the p53 "κB motif" oligonucleotides.


Target genes for NF-κB and their "κB" motifs.

human IL-8 promoter		
5'-GGAATTTCCTC-3'		
human p53 promoter		
5'-TGGGGTTTTCCCC-3'		
mouse p53 promoter		
5'-TGGGACTTTCCCC-3'		
Inhibitors of NF-κB/IκB protein are		
Class I	NFKN1	p105
	NFKN2	p100
and IκB proteins which contain ankyrin repeats.		
IκBα S32D		

METHODS

Human monocytes or U937 monocytic cells were cultured in $6x10^6$ cells / 3 ml medium, DMEM added 25 mM Hepes and 5% FCS, (Hyclone, logan, UT). Cells were then either non stimulated or stimulated for 24 hours with 10 ng/ml of rIL-10 or equimolar amounts of IT9302 or its analogues. The following day cells were stimulated with rIL-10 or analogues once more for 30 minutes before stimulation with 30 ng/ml of IL-1β for 1 hour.
Peptides were designed and synthesized by professor Arne Holm, Royal Veterinarian Academy, Copenhagen. The peptides were dissolved in sterile saline before used.
Cells were washed in ice cold PBS and nuclear proteins were isolated as previously described (Johansen et al., 2000).
Electrophoretic mobility shift assay (EMSA) for NF-κB binding was performed with 2 µg of nuclear protein added 2 µl of 32P-labelled NF-κB probe:
IL-8 consensus NF-κB 5'-CAAATCGGGAATTTCCTC-3' or Promega consensus NF-κB 5'AGTTGAGGGGACTTTCCCAGGC-3' were purchased from AH diagnostic, Aarhus, Denmark.
Supershift reactions included anti-NF-κB/p65 antibody and anti-NF-κB/p50 antibody (Santa Cruz, CA).

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RESULTS

We compared two synthetic IL-10 analogues A2 and A3, which are nona-peptides containing non-natural amino acids, with IL-10 and IT9302 for their ability to block NF-κB binding to specific "κB motifs" (Fig.2-5).
In a system of human monocytes, IT9302 and A3 more efficiently suppressed NF-κB binding to the IL-8 "κB motif" than to the p53 "κB motif" (Fig.2 and 3). Also, in repeated experiments, A3 was more potent than both recombinant IL-10 and the nonapeptide IT9302. In fact A3 suppressed the binding to levels below that of the negative control.
In U937 cells, the relative NF-κB binding was also suppressed by IL-10, IT9302 as well as the two new analogues (equivalent to 10 ng/ml of rIL-10) in a dose dependent manner.
A3 and A2 analogues were more efficient than IL-10 and IT9302 with respect to inhibiting the binding to the IL-8 "κB motif" while A3 was most efficient with respect to inhibition of the binding to the p53 "κB motif" in U937 cells.

DISCUSSION & CONCLUSION

IκB-α can specifically blocksNF-κB/p65 binding to DNA Ghosh S., Baltimore D. (1990) and thereby block binding of p65/65 homodimers to IL-8 "κB motif". In our hands, effective de novo synthesis of IκB-α appears to be dependent on prolonged (overnight) stimulation with IL-10 or its analogues according to our studies. On the other hand, blocking of NF-κB p50/p65 by p50/p50 may occur within 30 minutes. We therefore stimulated the cells twice and this could be of importance for an effective suppression of NF-κB binding to DNA.
In this study we compare the effect of IL-10 and its synthetic analogues with respect to their modulary capacity on the binding of NFkappaB to specific motifs, namely the p53 and IL-8 motifs. We find that:

- *IL-10 significantly modulate the binding of NF-kappa to the p53 and the IL-8 motifs.*
- *The C-terminal domain of IL-10, represented by IT9302 appears to be responsible for this effect.*
- *Structural modifications of IT9302 may improve the potency of this IL-10-like effect.*
- *Thus, the new IL-10 analogues, which are structural modifications of IT9302 appears to overcome previous stability problems, which are attributed to IT9302 (data not shown). This enhanced stability could explain the increased potency of the new analogues compared to IL-10 and IT9302.*

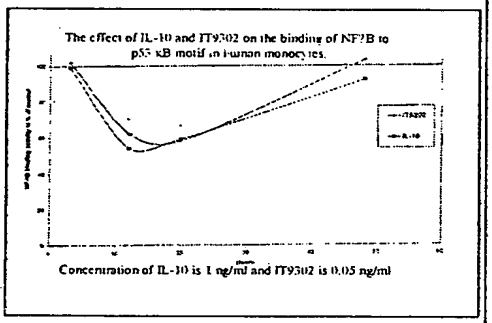

FIG. 1

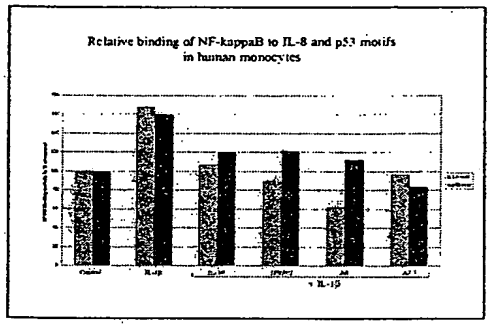

FIG. 2

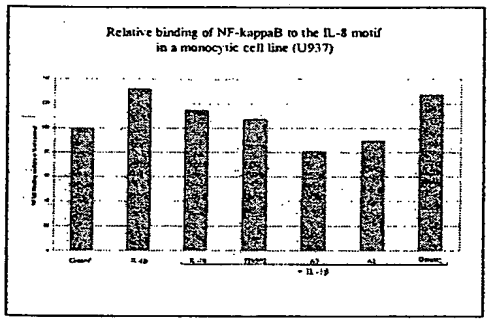

FIG. 3

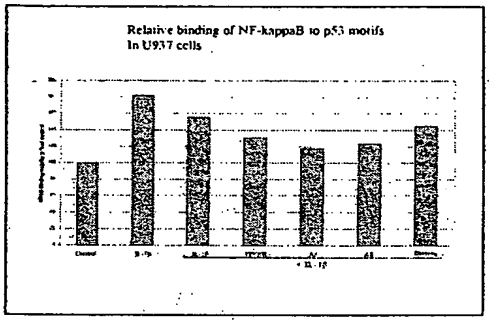

FIG. 4

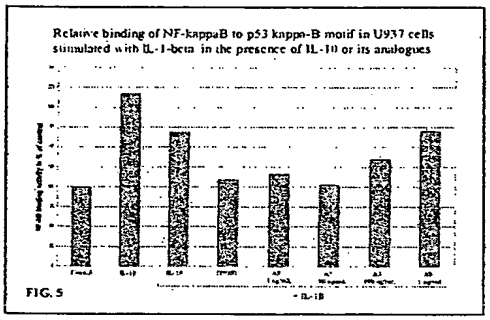

FIG. 5